

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER

1406/50

U.S. APPLICATION NO. (If known, see 37 CFR 1.5

10/088853

INTERNATIONAL APPLICATION NO.  
PCT/EP00/09217INTERNATIONAL FILING DATE  
20 September 2000 (20.09.00)PRIORITY DATE CLAIMED  
21 September 1999 (21.09.99)TITLE OF INVENTION  
METHOD FOR IDENTIFYING MHC-RESTRICTED ANTIGENSAPPLICANT(S) FOR DO/EO/US GSF-FORSCHUNGSZENTRUM FÜR UMWELT UND GESUNDHEIT, GMBH; ARTEMIS  
PHARMACEUTICALS GMBH; BORNKAMM, Georg W.; HOBOM, Gerd; MAUTNER, Josef and NIMMERJAHN, Falk

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☒ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

## Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13.1 and 35 U.S.C. 1.821-1.823.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:

Express Mail" mailing number EV023032219 US

Date of Deposit 21 March 02

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Shay E. Dunn

Copy of WO 01/22083; copy of PCT/IB/304; copy of PCT/ISA/220; copy of PCT/RO/101; copy of PCT/IPEA/416; English translation of claims as amended under PCT Rule 66.4;

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10/088853	PCT/EP00/09217	1406/50

21. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
<b>BASIC NATIONAL FEE (37 CFR 1.492 (a)(1)-(5)):</b>					
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . .				\$1040.00	
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International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . .				\$100.00	
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Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	0.00
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	\$	
Total claims	17 - 20 =	0	x <b>\$18.00</b>	\$	0.00
Independent claims	1 - 3 =	0	x <b>\$84.00</b>	\$	0.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ <b>\$280.00</b>	\$	280.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$	1,170.00
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	585.00
<b>SUBTOTAL =</b>				\$	585.00
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	0.00
<b>TOTAL NATIONAL FEE =</b>				\$	585.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +				\$	0.00
<b>TOTAL FEES ENCLOSED =</b>				\$	585.00
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**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:  
Richard E. Jenkins  
JENKINS & WILSON, P.A.  
Suite 1400 University Tower  
3100 Tower Boulevard  
Durham, nc 27707  
US



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*Richard E. Jenkins*  
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Richard E. Jenkins  
NAME  
28,428  
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Shay E. Dunn



10/088859  
JC10 Rec'd PCT/MPO 21 MAR 2002

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Bornkamm et al.

Group Art Unit: Not Assigned

**Serial No.: Not Assigned**

Examiner: Not Assigned

Filed: Herewith

Docket No.: 1406/50

For: METHOD FOR IDENTIFYING MHC-RESTRICTED ANTIGENS

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PRELIMINARY AMENDMENT

Honorable Commissioner for Patents  
BOX PCT  
Washington, D.C. 20231

Dear Sir:

Kindly amend the subject application as follows:

IN THE SPECIFICATION:

Please insert the paragraph heading on page 1 of the English translation of the subject application, before the title, as follows:

--Description--

Please insert the paragraph heading on page 1 of the English translation of the subject application, before the first full paragraph, as follows:

--Technical Field --.

Please insert the paragraph heading on page 1 of the English translation of the subject application, before the second full paragraph, as follows:

--Background Art --.

Please insert the paragraph heading on page 5 of the English translation of the subject application, before the first full paragraph, as follows:

--Summary of the Invention--.

Please insert the paragraph heading on page 5 of the English translation of the subject application, before the second full paragraph, as follows:

--Brief Description of the Drawings--.

Please insert the paragraph heading on page 6 of the English translation of the subject application, before the third full paragraph, as follows:

--Detailed Description of the Invention--.

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P12541

## METHOD FOR IDENTIFYING MHC-RESTRICTED ANTIGENS

Two findings from basic research form the rational basis of present tumor immunology: 1. tumor development is caused by genetic alterations of the cell which result in the expression of abnormal gene products; 2. T cells are capable of recognizing such changes in the protein pattern of malignant cells. A prerequisite for the induction of an anti-tumor immune response is the activation of antigen-specific T cells. The identification of T cell tumor antigens on a molecular basis therefore creates the basis for the development of antigen-specific vaccines as well as for other forms of T cell-mediated immune therapies (Rosenberg, 1996, 1999).

In recent years several antigens have been identified, particularly for the malignant melanoma, which are recognized by autologous T cell of the patients. A possible therapeutic benefit of these antigens is currently evaluated in the context of clinical studies. For a broad clinical use, however, it would be necessary to identify as many tumor antigens as possible because: 1. the antigens known up to now generally are expressed only in a small percentage of all malignant melanomas and thus are only useful in a small patient collective; 2. since antigens are presented by HLA molecules and since HLA are highly polymorphic among the human population it is necessary to identify as many antigens

as possible so that antigens will be available for the vaccination of any HLA constellation; 3. vaccinations carried out with only one antigen frequently result in the neutralization of this antigen by the tumor and thus in the development of resistance. This resistance development is to be prevented by simultaneous vaccination with as many antigens as possible. Therefore, the identification of other tumor antigens in the case of melanoma as well as other tumors is a necessary prerequisite for successful immune therapies.

On an experimental level, the identification of tumor antigens is composed of two steps. First the isolation of tumor-specific T cells of the patient by repeated in vitro stimulation with autologous tumor cells, and second the identification of the antigens recognized by the T cells on a molecular level. For this purpose, a simple and widely useful method would be desirable.

Several methods for identifying MHC-restricted T cell antigens are known from the prior art. Known approaches particularly include the following methods (Rosenberg, 1999): Transient transfection of allogenic or xenogenic cell lines; elution and HPLC fractionation of MHC-bound peptides; retroviral transduction of autologous fibroblasts.

These methods and the disadvantages which accompany them will be detailed in the following.

1. Transient transfection of allogenic/xenogenic cell lines  
This method is based on the expression of cDNA libraries from tumors in established cell lines (Boon, 1993). As the target cells for this purpose serve 293 or COS-7 cells which can be

transfected very efficiently. With respect to the HLA genotype of the patient in question, however, the cell lines used are allogenic (293) or xenogenic (COS-7) cell lines, respectively. Because T cells are MHC-restrictive, i.e. recognize their antigen only in the complex with the correct MHC molecule, knowledge of the restriction elements is an absolute requirement for the identification of antigens. T cell recognition of the antigens introduced is only possible by co-transfection of the corresponding restriction element. However, in most of the cases the identification of the appropriate restriction element is difficult or even impossible. Since the target cells used are also MHC class II-negative, this method is limited to the identification of MHC class I-restricted antigens wherein the restriction element is known.

## 2. Biochemical identification of the antigen

In addition to expression cloning, an identification of MHC class I-restricted tumor antigens can also be performed in a biochemical manner (Cox et al., 1994). For this purpose, tumor cells are lysed and the restrictive MHC molecules are subjected to immune precipitation using monoclonal antibodies. From these MHC molecules the bound peptides are eluted and separated by means of reverse phase HPLC. Subsequently, the target cells are loaded with individual peptide fractions. These target cells are characterized by expressing only the corresponding restriction element in a form not bound to peptide. Exogenous addition of peptides therefore results in rapid binding by the 'empty' MHC molecules. By co-cultivation of these peptide-loaded target cells with tumor-specific T cells the positive fractions are identified and the antigen is determined by means of the peptide sequence. As mentioned under 1, also in this case

knowledge of the HLA restriction element is an absolute requirement for the identification of T cell antigens, i.e. both for the immune precipitation of the restrictive MHC molecules, and for loading of the correct target cells with the eluted peptides. Another disadvantage is the enormously high amount of tumor required for isolation of the peptide so that this method may be only used for tumors which can be cultured and expanded easily in vitro. Identifying T cell antigens in this method is also limited to MHC class I-restricted antigens since an analogous HPLC fractionation of MHC class II peptides is impeded due to their length polymorphism.

### 3. Retroviral transduction of autologous fibroblasts

The problem described above of required identification of the restriction element is overcome if autologous target cells are used for the expression of the cDNA library from the tumor. Large amounts of cells are required for carrying out an antigen screening so that only those cells of the patient may be used as the target cells which can be expanded in vitro to an appropriate extent. Fibroblasts may be obtained from small skin biopsies and in addition may be easily transduced by retroviruses. Identification of MHC class I-restricted antigens by retroviral transduction of autologous fibroblasts with tumor-derived cDNAs therefore has been introduced as a method which overcomes the requirement to define the restriction element (Wang et al., 1998). However, this method is accompanied by several disadvantages. Primary fibroblasts may be cultured in vitro only for a limited number of passages. Moreover, since fibroblasts lack expression of MHC class II this method is also limited to the identification of MHC class I-restricted antigens. Compared to transient expression cloning as described under 1 the

retroviral transduction of target cells is much more laborious. The main disadvantage of this method, however, is the relatively low expression of the genes introduced by the retrovirus. This low level of expression results in an about 10 times reduced sensitivity and thus in a screening effort which is 10 times higher compared to that of transient transfection.

It is an object of the invention to provide a method avoiding the above-mentioned disadvantages of the prior art and which particularly does not require knowledge of the restrictive MHC molecule, which enables an unlimited proliferation of the target cells, and at the same enables the identification of both MHC class I and MHC class II-restricted antigens.

According to the invention, this object has been achieved by the method according to claim 1. Preferred embodiments of the invention become clear from the dependent claims, the following description as well as the Examples and Figures. The Figures show:

Figure 1: LCL infection with influenza

For determining the influenza infection rate of LCLs the cell line LCL1.26 was incubated with recombinant influenza viruses (FPV-1104 as vector) which carry the gene for the green fluorescence protein under the control of a promoter characterized by an enhanced activity as compared to the wild type promoter (promoter-up variant). After 24 hours the green fluorescent cells were counted under UV light.

Figure 2: Presentation of the model antigen in the context of MHC class II molecules after infection with recombinant influenza virus. LCL1.11 were infected by wild type (wt) or



recombinant FPV influenza virus, respectively, expressing the model antigen under the control of the promoter-up variant. After viral transduction of the model antigen (MA) into LCL1.11, GM-CSF secretion occurs by the model antigen-specific T cell clone but not after infection with the wild type virus (wt) or after infection of influenza virus carrying the GFP gene.

Figure 3: LCL infection with retroviruses

1 x 10<sup>5</sup> cells of the EBV-immortalized lymphoblastoid cell line LCL1.26 were infected with recombinant retrovirus expressing the green fluorescence protein. 72 hours following infection, the green fluorescent cells were counted.

Figure 4: Presentation of the model antigen in the context of MHC class II molecules after infection with recombinant retrovirus.

1 x 10<sup>5</sup> cells of the lymphoblastoid cell line LCL1.11 were subjected to retroviral transduction with the neomycin phosphotransferase II gene (Pinco-NeoR) or the green fluorescence protein gene (Pinco-GFP, Grignani et al., 1998), respectively. 72 hours later, the infected and not infected LCL1.11 cells were each incubated with 1 x 10<sup>5</sup> T cells. The MHC class II-restricted T cells used are specific for an epitope of the neomycin phosphotransferase II protein presented on HLA-DP3. After 24 hours of co-cultivation of the cells, the GM-CSF concentration in the cell supernatant was determined by means of an ELISA.

Thus, the present invention creates a method for identifying MHC-restricted antigens, i.e. of both MHC class I and/or class II-restricted antigens. In this respect, the following steps are also comprised by the method of the invention:

- The object underlying the invention is to isolate the mRNAs from a cell, for example an animal or human tumor cell, and to use these mRNAs for the preparation of a cDNA library. Analogously, the cells may be cells infected with a



microorganism such as a bacterium, a virus, a fungus, or a protozoan. It should be understood that also cells carrying mixed infections may be used. Alternatively, instead of preparing a cDNA library from the infected cell in these cases also a gene library may be used which is prepared directly from the microorganism to be studied. In a following step, the cDNA or DNA of the gene library is introduced into the genome of retroviruses or as additional vRNA into modified influenza viruses generating recombinant retroviruses or influenza viruses, respectively. The retroviruses used are preferably amphotrophic or pseudotyped retroviruses. The preparation of recombinant retroviruses as well as examples of amphotrophic viruses are described in Kinsella et al. (1996), examples for pseudotyped retroviruses are described in Miletic et al. (1999). Another example of retroviruses is the group of lentiviruses, particularly HIV and SIV.

The modified influenza viruses used preferably are promoter-up variants of FPV Bratislava. The preparation of such influenza viruses having promoter-up variants is described in Neumann and Hobom (1995), Flick and Hobom (1999) as well as in WO-A-96/10641.

The above-mentioned influenza promotor-up variants show an enhanced transcription rate (both with the vRNA promoter and in the cRNA promoter of the complementary sequence) as well as an enhanced replication and/or expression rate relative to the wild type and are different from the wild type in that they contain at least one segment (originally present or additional) in which a total of up to 5 nucleotides are replaced in the 5' and 3' conserved regions of the wild type. Preferably, the nucleotides in positions 3 and 8 (counted

from the 3' terminus) are replaced by other nucleotides in the 3' conserved region of the wild type consisting of 12 nucleotides wherein then only the nucleotides introduced form a base pair (pos. 3: G, then pos. 8: C; pos. 3: G, then pos. 8: G; etc.). Moreover, also the nucleotide in position 5 of the 3' conserved region (as counted from the 3' terminus) may be replaced.

The 3' conserved regions of wild type influenza viruses have the following sequences:

influenza A: (5')-CCUGCUUUUGCU-3'  
 influenza B: (5')-NN(C/U)GCUUCUGCU-3'  
 influenza C: (5')-CCUGCUUCUGCU-3'

Optionally, there may be also made exchanges in the 5' conserved region of the wild type consisting of 13 nucleotides, e.g. in positions 3 and 8 again with the proviso that the inserted nucleotides form a base pair. The 5' conserved regions of wild type influenza viruses have the following sequences:

influenza A: (5')-AGUAGAAACAAGG-3'  
 influenza B: (5')-AGUAG(A/U)AACA(A/G)NN-3'  
 influenza C: (5')-AGCAGUAGCAAG(G/A)-3'

Preferably, those mutants of the influenza virus are used in the present invention wherein the alterations G3A and C8U may be made in the 3' conserved region. Most preferable influenza mutants are the influenza A mutants and particularly those mutants which additionally show the U5C exchange (the above-mentioned mutants are numbered starting from their 3' ends; counting from the 3' end is indicated by a line above the

number such as G3A). Further preferred influenza mutants comprise the mutations G3C, U5C, and C8G (counted from the 3' end) in the 3' terminal nucleotide sequence resulting in the following 3' terminal nucleotide sequence:

(5')-CCUGGUUCUCCU-3'

Among the influenza viruses defined above those are particularly preferred which show the following 3' terminal nucleotide sequence:

(5')-CCUGGUUCUACU-3'

In the case of modified influenza A viruses, the modified segment preferably carries also the modifications U3A and A8U in its 5' terminal sequence, and in the case of influenza C viruses it may additionally have the modifications C3U and G8A in its 5' terminal sequence.

The most preferred influenza promoter-up variants of the present invention have the following general structures:

influenza A (promoter-up variant '1104'):

5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>...(880-2300 ntd)...N'N'N'CCUGUUUCACU-3'

influenza A (promoter-up variant '1920'):

5'-AGAAGAAUCAAGGNNNU<sub>5-6</sub>...(880-2300 ntd)...N'N'N'CCUGUUUCACU-3'

influenza A (promoter-up variant '1948'):

5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>...(880-2300 ntd)...N'N'N'CCUGGUUCCCU-3'

influenza B:

5'-AGUAG (A/U) AACA (A/G) NNNNNNU<sub>5-6</sub>...(880-2300 ntd)...N'N'N'N'N' (C/U) GUUUCACU-3'

influenza C:

5' -AGUAGUAACAAAG (G/A) GU<sub>5-6</sub>... (880-2300 ntd) ...CCCCUGUUUCUACU-3'

The indications in these structures have the following meanings:

- 1) underlined (and bigger): the mutations of the wild type sequence required to generate the promoter-up variant;
- 2) bigger A, not underlined, in the 5' portion of the sequences: additional A (position 10) forming an angle;
- 3) (A/G) at a particular position: different isolates or individual segments, respectively, having different sequences at positions which may be analytically exchanged;
- 4) N and N': positions which are not defined but form base pairs, complementary between 5' and 3' end, different for individual of the eight segments but the same for all isolates;
- 5) (880-2300 ntd): the segment length of the viral segments, in the case of segments containing foreign genes, however, up to 4000 ntd.

The recombinant viruses are propagated in suitable host cells and are optionally isolated by methods known per se. Using these recombinant retroviruses or influenza viruses, respectively, immortalized autologous cells are then infected, preferably B cells or dendritic cells which express MHC class I and/or MHC class II molecules. Afterwards, the steps d) to g) as described above are carried out.

According to the invention, 'autologous' refers to the origin of the cells, i.e. the cells originate in the same individual as the T cells used for screening for antigen expression.

Naturally, the present invention requires the use of T cell clones recognizing an antigen in particular cells. Since the identity of the antigen recognized is unknown, it is to be determined which antigen is recognized by the T cells. The availability of the T cell clones thus gives no indication as to the nature of the antigen. If, for example, T cell clones are used which recognize a prostate carcinoma line but not EBV immortalized cells or fibroblasts of the same individual then the aim would be to identify the yet unknown antigen by means of a cDNA library from the prostate carcinoma cells, the autologous EBV immortalized cells being the recipient cells for the cDNA library, and the specific T cell clones.

In the following the invention will be explained in detail.

A prerequisite for the present invention was the establishment of tumor-specific T cell clones. Since T cells recognize antigen only in the context of MHC molecules and an identification of the restrictive MHC molecules has been impossible in the case of some clones, it was impossible to employ already established methods for identifying T cell antigens for these clones. Since cells of the different tissues of an individual all express identical MHC molecules, a way was sought to utilize autologous B cells of the patient as recipient cells for the expression of tumor-derived cDNA libraries. B cells of any individual may be infected with the Epstein-Barr virus, a human member of the group of lymphocryptoviruses or related primate viruses of this group, may be immortalized and utilized in a practically unlimited amount in the form of so-called lymphoblastoid cell lines (LCLs). The use of autologous LCLs avoids identification of the MHC restriction elements which is sometimes difficult if not impossible. Since LCLs constitutively express MHC class I

and MHC class II, an identification is possible of both MHC class I-restricted antigens recognized by cytotoxic T cells and MHC class II-restricted antigens recognized by T helper cells.

The use of autologous LCLs of the patient as target cells, however, requires efficient gene transfer into these cells which up now has been achieved neither by chemical nor by physical transfection procedures. Relatively little is known about the infection rate of LCLs with viruses. As detailed in the following, the use of modified influenza A virus on the one hand leads to LCL infection with an efficiency which has never been reached to date, and on the other hand by using transcriptionally activated regulatory elements of the virus itself in the FPV virus variant an extraordinarily high gene expression of the genetic information introduced is achieved leading to a high sensitivity and thus simplicity of detection.

The use described herein of influenza A virus-derived vectors for gene transfer into LCLs required the determination of the efficiency of infection. As shown in Figure 1, up to 80% of the lymphoblastoid LCL1.26 cell lines are infected with this viruses.

Several other requirements in addition to a high infection rate must be met in the intended use to enable utilization of the influenza virus system for the above-mentioned purpose.

1. Expression level of the foreign gene introduced?

A high expression rate of the foreign sequence introduced into the cells is essential for a high sensitivity of the method of detection. By using the transcription/translation



machinery of influenza virus, the expression rate was increased by 5 times as compared to transient transfection and the expression level was about 10 times higher as in retroviral transduction, as determined by Western analyses for a model antigen.

2. Virus inference with antigen presentation and recognition?

It has been known for several viruses that they interfere with antigen presentation and thus escape recognition by the immune system. To exclude analogous mechanisms for the influenza A virus used a gene encoding a model antigen was introduced into the FPV viruses and used to infect LCLs. MHC presentation of the model antigen was determined by means of a model antigen-specific T cell clone. As shown in Figure 2, influenza virus does not interfere with antigen presentation. Moreover, the influenza virus infection neither results in unspecific T cell activation nor in cytokine release by infected LCLs.

3. T cell infection?

To detect T cell activation requires co-cultivation of target cells and T cells for at least 20 hours. A possible infection of T cells by the influenza virus used (from the supernatant or released from LCLs) together with lysis of the cells which starts 8 hours later would impede the detection of specific T cell activation. As confirmed also by means of the green fluorescence protein (GFP), the influenza virus used does not infect or at least not productively infect the T cells, i.e. no viral genes are expressed.

To determine the influenza infection rate of LCLs, cell line LCL1.26 was incubated with recombinant influenza virus

carrying the gene for green fluorescence protein. After 24 hours the green fluorescent cells were counted under UV light.

A possible alternative for gene transfer by recombinant influenza viruses are recombinant retroviruses which therefore were included in our studies. As shown in Figure 3, a very good gene transfer efficiency could be achieved also using recombinant retrovirus. By means of model antigens, an antigen-specific T cell stimulation could be detected also using recombinant retrovirus (Figure 4). However, retrovirus does not achieve the same high expression level as does influenza virus.

In the following, the invention will be explained in general.

The starting point of the method according to the invention is the isolation of mRNA or DNA from cells carrying antigens to be subjected to examination. For this purpose cells of human origin are of particular use but cells are also useful which are derived from animals, for example rodents such as mice or rats. Preferably these cells are derived from a patient suffering from a tumor, e.g. a tumor of the hematopoietic system such as a B cell tumor, for example a leukemia. However, the method may be employed also for identification of self antigens or foreign antigens, e.g. from tissue carrying microbial infections (bacteria, viruses, fungi, protozoans as well as any possible combined infection). In the case of a known microorganism, its genetic information may also be used directly (in the form of RNA or genomic DNA).

Isolation of the mRNA or DNA is carried out by methods of molecular biology known per se. In this respect, see for

example Sambrook et al., (1989). The mRNA is then transcribed into cDNA also using known techniques to obtain a cDNA library of the cell. In this respect, also see the reference cited above.

The genetic information included in the mixture of cDNA or DNA fragments is then introduced into the influenza virus genome. Generally, the procedure will be as follows:

The genetic information of the proteins encoded by influenza A viruses are contained on 8 negative strand RNAs wherein each of the coding regions is flanked by virus-specific promoter and terminator sequences regulating the transcription and replication as well as the packaging of the vRNAs into virus particles.

The preparation of recombinant influenza viruses, i.e. packaging of a foreign gene into influenza A virus particles and expression of this gene after infection of a target cell requires first that this gene is present as a negative strand RNA and second that it carries the viral promoter and packaging signals. This may be achieved for example by using a plasmid vector which carries a resistance gene such as for ampicillin and a bacterial origin of replication and in addition a polylinker sequence immediately flanked on both sides by the non-translated (cDNA) promoter sequences of influenza A virus, however, in a modified form which results in an enhanced promoter activity. The viral 5' and 3' promoter sequences themselves are flanked by the sequences of the human RNA polymerase I promoter and terminator isolated from human rDNA.

To prepare a cDNA library, the cDNAs are cloned from the tissue to be examined into the polylinker sequence of this plasmid in an inverse orientation with respect to the RNA polymerase I promoter and are amplified in *E. coli*. After transient transfection of these plasmids into suitable target cells, the transcriptional activity of RNA polymerase I from the cDNAs introduced results in pseudoviral negative strand RNAs having viral transcription and packaging signals at their ends. Because the packaging of viral RNAs into virus particles is not dependent on the coding region but exclusively from the regulatory signal sequences on the 5' and 3' ends, a superinfection of the transfected cells with influenza A helper virus (FPV Bratislava) will result in incorporation of these pseudoviral RNAs into the newly formed virus particles released into the culture supernatant. The viruses released into the cell culture supernatant are harvested and optionally further concentrated. It should be understood, however, that also other vectors known to those skilled in the art or to be constructed by those skilled in the art in the frame of the experiments based on influenza viruses but also on the retroviruses described below may be used in the context of the invention.

In a next step, autologous B cells are infected with the recombinant virus particles containing one or more copies of cDNAs in the form of negative strand vRNAs. Prior to infection the B cells must be immortalized wherein preferably EBV genes are used. However, also other immortalizing systems are known, for example oncogenes. The immortalizing procedure is as follows:

Lymphocytes (PBLs) are purified from the peripheral blood of the donor using a Ficoll gradient and incubated with EBV-

containing cell culture supernatant e.g. from the cell line B95-8. This simian cell line releases infectious EBV into the cell culture supernatant whereby the B cells of the donor are infected and immortalized.

For disclosure, reference is made for example to the following publications: v. Knebel Doeberitz et al., 1983; Rickinson et al., 1984.

Infection of the immortalized autologous B cells results in an expression of the pseudoviral gene segments derived from the original cell which are expressed in the form of proteins similar to the genetic information of own negative strand RNAs of the virus. Cleavage products of these proteins are presented by the antigen presentation machinery of the cell in the context of MHC molecules on the cell surface of the B cells where they can be recognized by antigen-specific T cells.

Thus, the present invention involves the transient introduction of mixtures of unknown cDNAs into the immortalized autologous cells capable of antigen presentation (APCs = antigen presenting cells) for the purpose of antigen identification. A stable transfection of the APCs with the cDNAs cannot be performed with the method according to the invention for two reasons. First, stable transfection by means of resistance genes always requires selection for growth-promoting and against growth-inhibiting genes. Because of the prolonged culture period of the cells due to selection this results in a shift in the peptides represented in the cDNA library. Second, expression of toxic, e.g. apoptose-promoting genes, leads to the death of transfected cells and therefore to the loss of such genes. To avoid these selective

mechanisms which would impede identification of potential antigens, the experimental conditions must be kept as short as possible. After transient transfection of cells with plasmids the maximum expression level is reached after 48-72 hours. Generally, for example the influenza system is superior in this respect to the typically employed transient transfection using plasmids. Due to the own transcription machinery of the virus, maximum expression of the foreign gene introduced is achieved already after 6-12 hours following infection. This serves to shorten the experimental periods after introduction of the foreign gene from about 72 hours to only 24 hours.

In a next step, therefore, the B cells infected with recombinant influenza virus are co-cultured with autologous T cell clones having a specificity for the antigen to be identified. If a cDNA was introduced and expressed as vRNA within the B cells by the influenza virus which encodes the antigen recognized by the T cells, the antigen-specific T cells are stimulated. Stimulation of the T cells via their T cell receptor is accompanied by the release of cytokines which may be detected by known methods such as ELISA procedures. It should be understood, however, that also other methods may be used which are capable of detecting antigen-specific T cell stimulation. In addition to the detection of cytokine expression these also include the detection of T cell-mediated cytotoxicity and other measurable parameters of T cell activation.

If the influenza virus population included virus particles encoding the antigen in question and were able to stimulate T cells to release cytokines the virus population is divided into smaller pools, and these are used for a repeated B cell

infection and antigen recognition procedure. After isolation of the viruses encoding the antigen, a following step may comprise the isolation and identification of the antigen recognized by the T cells.

Alternatively, instead of modified influenza viruses there may also be used retroviruses for infection of autologous LCLs and expression of the antigens to be identified. Retroviruses achieve a good efficiency of transduction similar to that of modified influenza viruses but their level of expression is not as high as that achieved with modified influenza viruses. The preparation of recombinant retroviruses is detailed in the prior art, and reference is made herein exemplarily only to the publication of Kinsella et al. (1996).

The following contains a description of two experiments as performed in the laboratory using on the one hand influenza virus '1104' and on the other hand retroviruses.

Preparation of recombinant influenza virus, infection of autologous LCLs and recognition of a model antigen

The open reading frame of the neomycin phosphotransferase II gene was inserted into the polylinker sequence of the influenza vector plasmid described above in a reverse orientation with respect to the polymerase I promoter and was used for transformation of E. coli. Antigen-specific T cells which recognize the neomycin phosphotransferase II gene product are available in our laboratory. After plasmid preparation using commercial methods (Quiagen Maxi Prep Kit), 5 µg of plasmid DNA was mixed with 185 µl of medium and 15 µl Lipofectamine TM (Gibco BRL) and incubated for 30 min at room

temperature. After addition of further 3 ml of medium the mixture was added to  $2.5 \times 10^6$  293 T cells. After 6 hours the supernatant was removed and replaced by cell medium. 18 hours following transfection the cells were washed and superinfected with influenza A virus (MOI = 1), and after incubation for further 15 hours the recombinant virus was harvested from the cell supernatant. To increase the virus titer,  $1 \times 10^7$  MDCK cells were incubated with 1 ml of virus supernatant. 10  $\mu$ l of this culture supernatant harvested after further 15 hours were used to infect  $1 \times 10^5$  LCLs which were then co-cultured with the same number of antigen-specific T cells. After 20 hours the GM-CSF concentration was determined in the supernatant using an ELISA.

Preparation of recombinant retroviruses, infection of autologous LCLs and recognition of a model antigen by antigen-specific T cells

Again, the neomycin phosphotransferase II gene was used as a model antigen for MHC class II restricted T cell recognition after retroviral transduction, and for detecting the infection efficiency the green fluorescence protein (GFP) was used. The open reading frames of both genes were introduced between the 5' and 3' retroviral long terminal repeats (LTRs) of Moloney murine leukemia virus (M-MuLV) in the plasmid PINCO (Grignani et al., 1998) and amplified in *E. coli* as described above. For the preparation of recombinant retroviruses, 5  $\mu$ g of plasmid DNA was mixed with 370  $\mu$ l of medium (without FCS) and 30  $\mu$ l Lipofectamine TM (Gibco BRL) and incubated for 30 min at room temperature. After addition of further 3 ml of medium the mixture was added to  $2 \times 10^6$  cells of the amphotropic packaging cell line Phoenix and incubated for 6 hours at 37°C. 24 hours after transfection, 2



$\mu\text{g}$  of puromycin/ml culture medium were added whereby only those cells survive which have incorporated the PINCO vector. After selection on puromycin for 48 hours, the cells were washed and resuspended in medium without puromycin. The culture supernatant containing virus was harvested after 48-72 hours, and the virus titer was  $2 \times 10^6$  viruses per ml of culture medium.

For infection of LCLs,  $1 \times 10^5$  cells were resuspended in 2 ml of virus supernatant, and polybrene was added in a concentration of  $2 \mu\text{g}/\text{ml}$ . Then the cells were centrifuged in a Varifuge 3.2S (Heraeus) at 1800 rpm for 30 min at room temperature. After addition of new virus supernatant and another centrifugation step the cells were incubated for 12 h at  $37^\circ\text{C}$ . Afterwards, the virus supernatant was replaced by culture medium. 48 hours following infection  $1 \times 10^5$  infected LCLs were co-cultured with the same number of antigen-specific T cells. After 20 hours the GM-CSF concentration was determined in the supernatant using an ELISA.

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**METHOD FOR IDENTIFYING MHC-RESTRICTED ANTIGENS**

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**C L A I M S**

1. Method for identifying MHC-restricted antigens comprising the following steps:
  - (a) preparing a gene library or a cDNA library from a cell or a microorganism to be studied;
  - (b) introducing the cDNA or the DNA of the gene library into the genome of retroviruses or as additional vRNA of modified influenza viruses which show an enhanced transcription, replication and/or expression rate as compared to the wild type to obtain recombinant virus particles;
  - (c) infecting immortalized autologous cells expressing MHC class I and/or MHC class II molecules on their surface with the recombinant virus particles obtained in step (b);
  - (d) expressing the proteins encoded by the cDNA or the DNA of the gene library within the autologous cells and presenting the cleavage products of these proteins generated by the autologous cell on the cell surface in a complex with MHC molecules;
  - (e) co-cultivating of T cells with the autologous cells;
  - (f) stimulating the T cells by such autologous cells presenting an antigen on their cell surface which is recognized by the T cells;
  - (g) isolating of the clones which express the antigen, and identifying the antigen.

2. Method according to claim 1 characterized in that the cell is selected from an animal or a human eukaryotic cell.
3. Method according to claim 1 or claim 2 characterized in that a tumor cell or a cell infected by a microorganism is used as the eukaryotic cell.
4. Method according to claim 3 characterized in that a cell infected by a virus or a bacterium or a fungus or a protozoan or from a combination of one or more of these microorganisms is used.
5. Method according to one or more of the preceding claims characterized in that negative strand RNAs derived from the cDNA or the DNA of the gene library are introduced into one or more segments of the modified influenza viruses and/or as an additional segment into the modified influenza viruses.
6. Method according to claim 5 characterized in that modified influenza A viruses are used as the modified influenza viruses and amphotropic or pseudotyped retroviruses as well as lentiviruses are used as the retroviruses.
7. Method according to claim 5 characterized in that the preparation of negative strand RNA is performed by transcription of the pseudoviral gene segments with RNA polymerase I.

8. Method according to one or more of the preceding claims characterized in that following step (b), by means of selection a concentration of the recombinant virus particles is performed and/or the recombinant virus particles are isolated.
9. Method according to one or more of the preceding claims characterized in that the cleavage products of the proteins are presented on the B cell in a complex with MHC class I or MHC class II.
10. Method according to one or more of the preceding claims characterized in that the stimulation of the antigen-specific T cells is measured by cytokine release, by T cell proliferation, or by detection of the cytotoxic activity of the T cells.
11. Method according to claim 10 characterized in that the release of cytokines is detected by means of an ELISA procedure.
12. Method according to one or more of the preceding claims characterized in that after introducing the cDNA or the DNA of the gene library a superinfection with wild type influenza virus is carried out.
13. Method according to one or more of the preceding claims characterized in that immortalization of the autologous cells is carried out using EBV genes or oncogenes.
14. Method according to one or more of the preceding claims characterized in that co-cultivation of the B cells is performed with T helper cells in the case of MHC class

II-restricted antigens and with cytotoxic T cells in the case of MHC class I-restricted antigens.

15. Method according to claim 1 characterized in that the gene library or cDNA library is prepared from a virus, a bacterium, a fungus, or a protozoan.
16. Method according to claim 1 characterized in that B cells or dendritic cells are used as the autologous cells.





**Figure 1**

Cell line	% green cells
LCL 1.26	~78

### Presentation of the model antigen in the context of MHC II

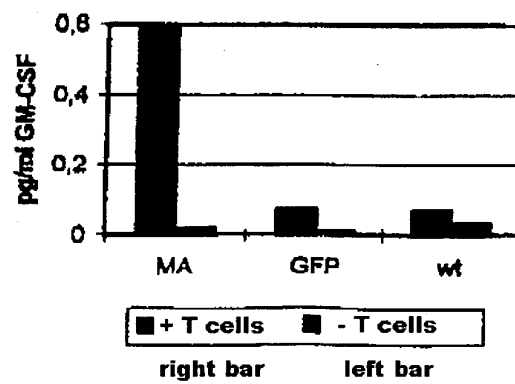


Figure3:

**LCL infection using retroviruses**

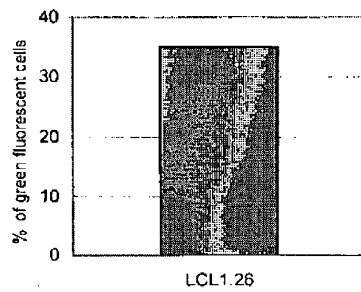
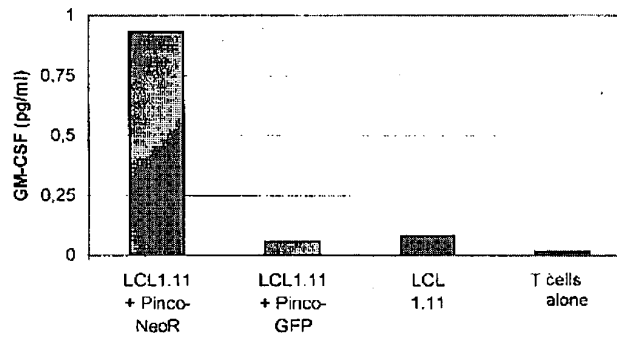


Figure 4:

**Presentation of the model antigen in the context of MHC class II molecules after infection with recombinant retroviruses.**



PCT/EP00/09217

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CLAIMS FOR ENTERING THE NATIONAL PHASE USA

1. Method for identifying MHC-restricted antigens wherein said method comprises the following steps:
  - (a) preparing a gene library or a cDNA library from a cell or a microorganism to be studied;
  - (b) introducing the cDNA or the DNA of the gene library as additional vRNA of modified influenza viruses which show an enhanced transcription, replication and/or expression rate as compared to the wild type to obtain recombinant virus particles;
  - (c) infecting (immortalized) antigen-presenting cells expressing MHC class I and/or MHC class II molecules on their surface with the recombinant virus particles obtained in step (b);
  - (d) expressing the proteins encoded by the cDNA or the DNA of the gene library within the infected antigen-presenting cells obtained in step (c) and presenting the cleavage products of these proteins generated by the infected cell on the cell surface in a complex with MHC molecules;
  - (e) co-cultivating of the infected antigen-presenting cells with T cells derived from the same organism as the infected antigen-presenting cells wherein the T cells are stimulated by antigen-presenting cells presenting an antigen on their cell surface which is recognized by the T cells;
  - (g) isolating of the clones which express the antigen, and identifying the antigen.

2. Method according to claim 1 wherein the cell to be studied is an animal or a human eukaryotic cell.
3. Method according to claim 2 wherein the eukaryotic cell is a tumor cell or a cell infected by a microorganism.
4. Method according to claim 3 wherein the cell is infected by a virus or a bacterium or a fungus or a protozoan or by a combination of one or more of these microorganisms.
5. Method according to claim 1 wherein the gene library or cDNA library is prepared from a virus, a bacterium, a fungus or a protozoan.
6. Method according to claim 1 wherein the negative strand RNAs derived from the cDNA or the DNA of the gene library are introduced into one or more segments of the modified influenza viruses and/or as an additional segment into the modified influenza viruses.
7. Method according to claim 1 wherein the modified influenza viruses are modified influenza A viruses.
8. Method according to claim 6 wherein the preparation of negative strand RNA is performed by transcription of the pseudoviral gene segments with RNA polymerase I.
9. Method according to claim 1 wherein following step (b), by means of selection a concentration of the recombinant virus particles is performed and/or the recombinant virus particles are isolated.

10. Method according to claim 1 wherein after introducing the cDNA or the DNA of the gene library a superinfection is carried out using wild type influenza virus.
11. Method according to claim 1 wherein immortalization of the antigen-presenting cells is carried out using EBV genes or oncogenes.
12. Method according to claim 1 wherein B cells or dendritic cells are used as the antigen-presenting cells.
13. Method according to claim 1 wherein the cleavage products of the proteins are presented on the B cell in a complex with MHC class I or MHC class II.
14. Method according to claim 1 or 13 wherein co-cultivation of the B cells is performed with T helper cells in the case of MHC class II-restricted antigens and with cytotoxic T cells in the case of MHC class I-restricted antigens.
15. Method according to claim 1 wherein stimulation of the antigen-specific T cells is measured by cytokine release, by T cell proliferation, or by detection of the cytotoxic activity of the T cells.
16. Method according to claim 15 wherein the release of cytokines is detected by means of an ELISA procedure.

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AMENDED CLAIMS ACCORDING TO RULE 66.4 PCT

1. Method for identifying MHC-restricted antigens wherein said method comprises the following steps:
  - (a) preparing a gene library or a cDNA library from a cell or a microorganism to be studied;
  - (b) introducing the cDNA or the DNA of the gene library as additional vRNA of modified influenza viruses which show an enhanced transcription, replication and/or expression rate as compared to the wild type to obtain recombinant virus particles;
  - (c) infecting (immortalized) antigen-presenting cells expressing MHC class I and/or MHC class II molecules on their surface with the recombinant virus particles obtained in step (b);
  - (d) expressing the proteins encoded by the cDNA or the DNA of the gene library within the infected antigen-presenting cells obtained in step (c) and presenting the cleavage products of these proteins generated by the infected cell on the cell surface in a complex with MHC molecules;
  - (e) co-cultivating of the infected antigen-presenting cells with T cells derived from the same organism as the infected antigen-presenting cells wherein the T cells are stimulated by antigen-presenting cells presenting an antigen on their cell surface which is recognized by the T cells;
  - (g) isolating of the clones which express the antigen, and identifying the antigen.

2. Method according to claim 1 wherein the cell to be studied is an animal or a human eukaryotic cell.
3. Method according to claim 1 or claim 2 wherein the eukaryotic cell is a tumor cell or a cell infected by a microorganism.
4. Method according to claim 3 wherein the cell is infected by a virus or a bacterium or a fungus or a protozoan or by a combination of one or more of these microorganisms.
5. Method according to claim 1 wherein the gene library or cDNA library is prepared from a virus, a bacterium, a fungus or a protozoan.
6. Method according to one or more of claims 1 to 5 wherein the negative strand RNAs derived from the cDNA or the DNA of the gene library are introduced into one or more segments of the modified influenza viruses and/or as an additional segment into the modified influenza viruses.
7. Method according to claim 1 or claim 6 wherein the modified influenza viruses are modified influenza A viruses.
8. Method according to claim 6 or claim 7 wherein the preparation of negative strand RNA is performed by transcription of the pseudoviral gene segments with RNA polymerase I.
9. Method according to one or more of claims 1 to 8 wherein following step (b), by means of selection a concentration of the recombinant virus particles is





16. Method according to claim 15 wherein the release of cytokines is detected by means of an ELISA procedure.
17. Use of modified influenza viruses having an enhanced transcription, replication, and/or expression rate as compared to the wild type for identifying MHC-restricted antigens.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES  
PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG(19) Weltorganisation für geistiges Eigentum  
Internationales Büro(43) Internationales Veröffentlichungsdatum  
29. März 2001 (29.03.2001)

PCT

(10) Internationale Veröffentlichungsnummer  
**WO 01/22083 A2**(51) Internationale Patentklassifikation<sup>7</sup>: G01N 33/53München (DE). NIMMERJAHN, Falk [DE/DE]; Dr.  
Pollmannstr. 6, 95349 Thurnau (DE).

(21) Internationales Aktenzeichen: PCT/EP00/09217

(74) Anwalt: BEHNISCH, Werner; Reinhard, Skuhra, Weise  
& Partner GbR, Postfach 44 01 51, 80750 München (DE).(22) Internationales Anmeldedatum:  
20. September 2000 (20.09.2000)(81) Bestimmungsstaaten (*national*): AE, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE,  
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PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ,  
UA, UG, US, UZ, VN, YU, ZA, ZW.

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:  
199 45 171.0 21. September 1999 (21.09.1999) DE  
199 51 543.3 26. Oktober 1999 (26.10.1999) DE  
199 62 508.5 23. Dezember 1999 (23.12.1999) DE(84) Bestimmungsstaaten (*regional*): ARIPO-Patent (GH,  
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), eura-  
sisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI,  
FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI-Patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE,  
SN, TD, TG).(71) Anmelder (für alle Bestimmungsstaaten mit Ausnahme  
von US): GSF-FORSCHUNGSZENTRUM FÜR  
UMWELT UND GESUNDHEIT GMBH [DE/DE];  
Ingolstädter Landstrasse 1, 85764 Oberschleissheim (DE).  
ARTEMIS PHARMACEUTICALS GMBH [DE/DE];  
Neurather Ring 1, 51063 Köln (DE).

## Veröffentlicht:

— Ohne internationalen Recherchenbericht und erneut zu  
veröffentlichen nach Erhalt des Berichts.

(72) Erfinder; und

(75) Erfinder/Anmelder (nur für US): BORNKAMM,  
Georg, W. [DE/DE]; Ottilenstr. 6a, 81243 München (DE).  
HOBOM, Gerd [DE/DE]; Arndtstr. 14, 35392 Giessen  
(DE). MAUTNER, Josef [DE/DE]; Grütznerstr. 6, 81667Zur Erklärung der Zweibuchstaben-Codes, und der anderen  
Abkürzungen wird auf die Erklärungen ("Guidance Notes on  
Codes and Abbreviations") am Anfang jeder regulären Ausgabe  
der PCT-Gazette verwiesen.

(54) Title: METHOD FOR IDENTIFYING MHC-RESTRICTED ANTIGENS

(54) Bezeichnung: VERFAHREN ZUR IDENTIFIZIERUNG VON MHC-RESTRINGIERTEN ANTIGENEN

(57) Abstract: The invention relates to a method for identifying MHC-restricted antigens.

(57) Zusammenfassung: Die vorliegende Erfindung beschreibt ein Verfahren zur Identifizierung von MHC-restringierten Antigenen.

PTO/SB/01 (10-01)

Approved for use through 10/31/2002. OMB 0651-0032

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Attorney Docket Number	1406/50
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1406/50

First Named Inventor	Bornkamm, Georg W.
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Bornkamm, Georg W.

**COMPLETE IF KNOWN**

Application Number 10 / 088,853

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Filing Date

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**Examiner Name**

**As the below named inventor, I hereby declare that:**

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original and first inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## METHOD FOR IDENTIFYING MHC-RESTRICTED ANTIGENS

(Title of the Invention)

the specification of which



is attached hereto

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**X**

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03/21/2002

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Application Number	10/088,853	and was amended on (MM/DD/YYYY)	(if applicable).
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10/088,853

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Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
PCT/EP00/09217	WIPO	09/20/2000	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
199 45 171.0	Germany	09/21/1999	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
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[Page 1 of 2]

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.									
NAME OF SOLE OR FIRST INVENTOR:				<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any]) <u>Georg W.</u>				Family Name or Surname <u>Bornkamm</u>					
Inventor's Signature <u>G. Bornkamm</u>				Date <u>13.5.02</u>					
Residence: City <u>München</u> <u>DEX</u>			State		Country <u>DE</u>		Citizenship <u>DE</u>		
Mailing Address <u>Otilostr. 6a</u>									
City <u>München</u>				State		ZIP <u>D-81243</u>		Country <u>Germany</u>	
NAME OF SECOND INVENTOR:				<input type="checkbox"/> A petition has been filed for this unsigned inventor					
Given Name (first and middle [if any]) <u>Gerd</u>				Family Name or Surname <u>Hobom</u>					
Inventor's Signature <u>G. Hobom</u>				Date <u>21.5.02</u>					
Residence: City <u>Giessen</u> <u>DEX</u>			State		Country <u>DE</u>		Citizenship <u>DE</u>		
Mailing Address <u>Arndtstr. 14</u>									
City <u>Giessen</u>				State		ZIP <u>D-35392</u>		Country <u>Germany</u>	
<input checked="" type="checkbox"/> Additional inventors are being named on the <u>1</u> supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.									

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PTO/SB/02A (11-00)

Approved for use through 10/31/2002. OMB 0851-0032

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# DECLARATION

## ADDITIONAL INVENTOR(S)

Supplemental Sheet

Page 1 of 1

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))

Family Name or Surname

Josef

Mautner

Inventor's  
Signature

Date 13.05.02

Residence: City München

State

Country DE

Citizenship DE

Mailing Address Grütznerstr. 6

Mailing Address

City München

State

ZIP D-81667

Country Germany

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))

Family Name or Surname

Falk

Nimmerjahn

Inventor's  
Signature

Date 13.08.02

Residence: City Thurnau

State

Country DE

Citizenship DE

Mailing Address Dr. Pollmannstr. 6

Mailing Address

City Thurnau

State

ZIP D-95349

Country Germany

Name of Additional Joint Inventor, if any:

☐ A petition has been filed for this unsigned inventor

Given Name (first and middle (if any))

Family Name or Surname

Inventor's  
Signature

Date

Residence: City

State

Country

Citizenship

Mailing Address

Mailing Address

City

State

ZIP

Country

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# SEQUENCE LISTING

<110> GSF-Forschungszentrum für Umwelt und Gesundheit  
Artemis Pharmaceuticals GmbH

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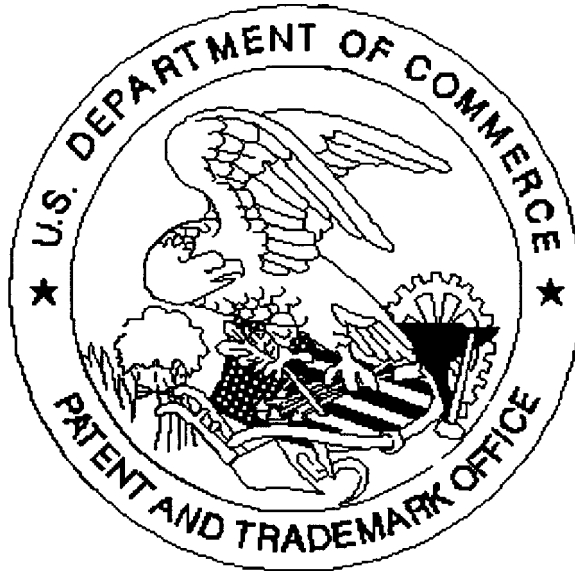
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*page 32, to 36, of specification are  
sequence listing*

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